

## Energy balance and liver respiratory activity in rats fed on an energy-dense diet

BY SUSANNA IOSSA, MARIA P. MOLLIKA, LILLÀ LIONETTI,  
ANTONIO BARLETTA AND GIOVANNA LIVERINI\*

*Department of General and Environmental Physiology, University of Naples "FEDERICO II",  
Naples, Italy*

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In the present study energy balance and liver respiratory activity were studied in rats fed on either a control diet or an energy-dense diet. Liver respiration was assessed both without added substrates and after the addition of hexanoate, glycerol, or sorbitol. The effect of ouabain on hexanoate-supported respiration was also determined. Metabolizable energy intake and energy expenditure increased in rats fed on an energy dense diet, but body-weight gain, as well as lipid and protein content, remained unchanged. When net energy expenditure, obtained excluding the total cost of storage, was expressed as a percentage of metabolizable energy, significant differences were found between the two groups of rats. This finding supports the presence of regulatory mechanisms in rats fed on an energy-dense diet, which are useful to counteract development of obesity. In addition, a significant increase in liver respiratory activity was found in rats fed on an energy-dense diet, both in the basal state and in that stimulated by added substrates. Na/K-pump-dependent O<sub>2</sub> consumption also increased in rats fed on an energy-dense diet. The results indicate that a greater production of metabolic heat by the liver can contribute to the increased energy expenditure found in rats fed on an energy-dense diet.

### Energy balance: Liver respiration: Fat intake

Many factors can influence energy expenditure and energetic efficiency. It is well known that starvation (Ma & Foster, 1986; Griggio *et al.* 1992) and food restriction (Garrow, 1986; Hill *et al.* 1988; Dulloo & Girardier, 1993) are characterized by reduced basal energy requirements, thus conserving body energy stores. On the other hand, hyperphagia may elicit a regulatory increase in energy expenditure, particularly in young rats, which therefore fail to exhibit excess weight gain (Rothwell & Stock, 1982). The regulatory increase in energy expenditure can be induced by varying dietary composition, for example by reducing the protein content of the diet (Rothwell *et al.* 1982), or by presenting rats with a cafeteria diet (Rothwell & Stock, 1982) and a high-fat low-carbohydrate energy-dense diet (Liverini *et al.* 1994, 1995; Iossa *et al.* 1995). It has been shown that brown adipose tissue and liver metabolic activities are increased in rats fed on low-protein (Tyzbir *et al.* 1981; Rothwell *et al.* 1982) and cafeteria (Berry *et al.* 1985; Rothwell & Stock, 1986; Ma *et al.* 1987) diets. We have previously shown an increase in FADH<sub>2</sub> oxidation and a decrease in NADH oxidation in hepatic mitochondria from rats fed on an energy-dense diet (Liverini *et al.* 1994; Iossa *et al.* 1995).

In view of these results, it appeared of interest to study liver respiratory activity in rats fed on an energy-dense diet. For this purpose, together with full energy balance

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measurements, we assessed the respiration under different conditions of stimulation in isolated hepatocytes derived from rats fed on an energy-dense diet. We also measured hepatic Na/K-pump-dependent O<sub>2</sub> consumption in rats fed on an energy-dense diet.

#### MATERIALS AND METHODS

Male Wistar rats with a mean initial body weight of about 80 g were chosen for the experiments. They were housed individually in grid-bottomed cages at 24° under an artificial circadian 12 h light–12 h darkness cycle. Two groups of ten rats were used. A control group (C) was fed *ad libitum* on a standard stock diet (Mucedola 4RF21, Settimo Milanese, Milan, Italy). The composition (% energy) of this diet was protein 29.0, lipid 10.6, and carbohydrate 60.4; its gross energy density was 15.88 kJ/g wet weight. The second group (ED) had free access to a composite energy-dense diet; the composition of this diet is reported in Table 1. This diet was characterized by a high fat content and by the presence of a meat component which is among the flavours most preferred by rats (Naim *et al.* 1985; Allard & LeBlanc, 1988). The experiment lasted 15 d. Animal care, housing and killing met the guidelines of the Italian Health Ministry.

At the end of the experimental period, five rats from each group were used for energy balance measurements and serum free fatty acid (FFA) and triacylglycerol determinations, while the other five rats were used for hepatocyte preparation.

#### *Energy balance measurements*

Body weights and feed intakes were monitored daily to allow calculations of body-weight gain and gross energy intake. The faeces were also collected daily for energy content measurements. The collected faeces were dried and ground to a powder before determining their energy content with a bomb calorimeter (Parr adiabatic calorimeter (Parr Instrument Co., Moline, IL, USA) calibrated with dry benzoic acid standard). The gross energy content of control and energy-dense diets was also determined by the bomb calorimeter.

Digestible energy intake (taking into account the feed spillage) was obtained by subtracting the energy measured in the faeces from the gross energy intake as measured from daily feed consumption. Metabolizable energy (ME) intake was expressed as

Table 1. *Composition of the energy-dense diet (g/kg)*

Component	
Control diet	280
Lyophilized meat*	395
Butter†	178
Alphacel (cellulose)	120
AIN 76 mineral mix‡	20
AIN 76 vitamin mix§	7
Gross energy density (kJ/g)	19.85
Percentage of energy from:	
protein	29
lipid	50
carbohydrate	21

\*Liomellin, STAR s.p.a., Milan, Italy, containing (in 10 g): 5.8 g protein (N × 6.25), 1.2 g lipid, 2.57 g carbohydrate, 0.2 g minerals, 0.2 g water.

†Lurpak, Denmark, locally purchased, containing 100 g water/kg.

‡American Institute of Nutrition (1977).

§American Institute of Nutrition (1980).

digestible energy intake  $\times 0.96$  (Barr & McCracken, 1984). The gain in energy was obtained by subtracting the energy content of an initial group (five rats killed at the beginning of the study) from that of each of the two experimental groups. At the end of the experiment the animals were anaesthetized by an intraperitoneal injection of chloral hydrate (400 mg/kg body weight), and blood was collected. Then, after gut content removal, the carcasses were autoclaved, chopped into small pieces, thoroughly mixed, and homogenized in water (final volumes equal to twice the carcass weight) with a Polytron homogenizer (Kinematica AG, Luzern, Switzerland). Samples of homogenates were desiccated into a dry powder from which small pellets (about 200 mg) were made. The energy content was measured with the bomb calorimeter. Energy expenditure was calculated from the difference between ME intake and energy gain.

#### *Circulating triacylglycerol and free fatty acid concentrations*

Serum samples were stored at  $-20^{\circ}$  until analysis. Triacylglycerols were measured using the lipase-glycerol kinase method, and FFA using the acyl-CoA synthetase-acyl-CoA oxidase method. The measurements were made using the enzymic kits obtained from Boehringer-Mannheim Biochemia, Milan, Italy.

#### *Preparation and incubation of liver cells*

Rat liver cells were prepared from 16-h-fasted rats as described by Seglen (1974), except that the rats were anaesthetized by an intraperitoneal injection of chloral hydrate (400 mg/kg body weight). The hepatocytes were washed and suspended in a medium containing 120 mM-NaCl, 5 mM-KCl, 50 mM-Hepes, 1 mM  $\text{KH}_2\text{PO}_4$ , 2 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgSO}_4$ , and 20 g/l fatty-acid-free bovine serum albumin. The final cell suspension was counted with trypan blue in a Burkner chamber in order to assess the viability (routinely  $> 90\%$ ).

Hepatocyte  $\text{O}_2$  consumption was measured polarographically with a Clark-type electrode maintained in a chamber at  $37^{\circ}$ . Portions corresponding to about  $10^6$  viable cells were incubated in the suspension buffer with different substrates, at the concentrations reported in Fig. 1.

#### *Statistical analysis*

The data are summarized using means with their standard errors of five different rats. Statistical significance between the means was examined by two-tailed Student's *t* test or by two-way ANOVA (only for main effects) followed by two-tailed Student's *t* test. Probability values less than 0.05 were considered to indicate a significant difference.

#### *Materials*

Collagenase (*EC* 3.4.24.3, type IV), hexanoate, glycerol, sorbitol, and ouabain were purchased from Sigma Chemical Co., St Louis, MO, USA. All other reagents were of the highest purity commercially available.

## RESULTS

Table 2 shows the results of body weight and carcass content measurements in C and ED rats. Initial body weight, final body weight, and weight gain did not differ significantly between the two groups. At the end of the experimental period, carcass lipid content was

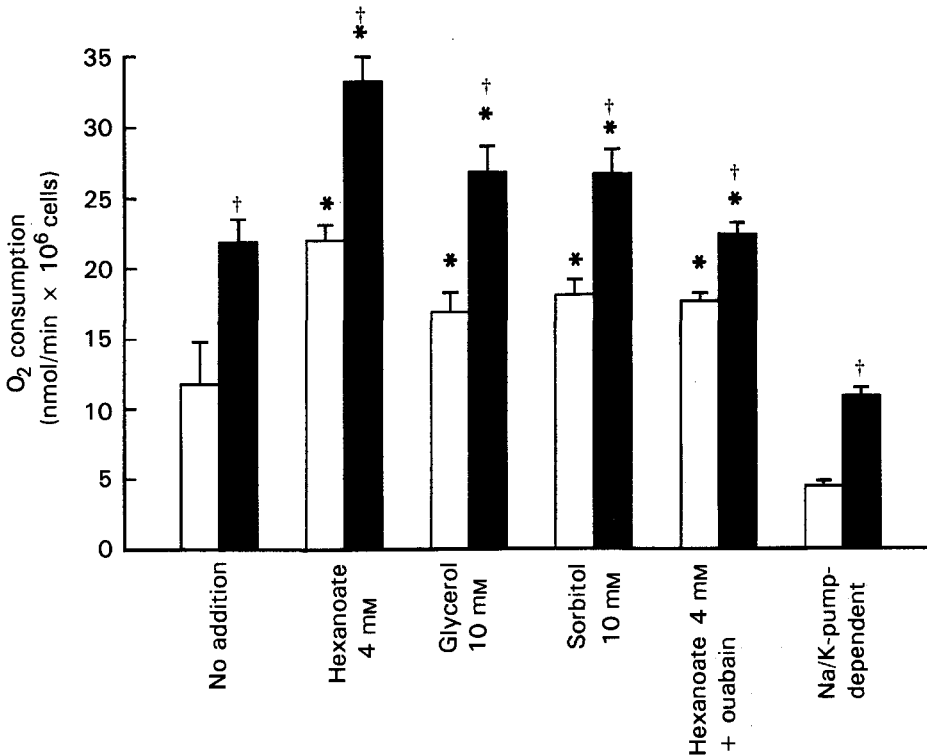


Fig. 1. Hepatocyte respiration in rats fed on control (□) and energy-dense (■) diets. Values are means with their standard errors represented by vertical bars for five rats. \*Significant effect ( $P < 0.05$ ) of addition (two-way ANOVA followed by two-tailed Student's  $t$  test). †Significant effect ( $P < 0.05$ ) of diet (two-way ANOVA followed by two-tailed Student's  $t$  test).

about 120 g/kg and carcass protein content was about 210 g/kg, both in C and ED rats. Table 2 also gives the results of energy balance measurements in the two groups of rats. The ME intake and energy expenditure of ED rats were significantly different (+30% and +51% respectively) from those of C rats, whereas the gain in body energy was not different between the two groups. Consequently, gross efficiency was significantly lower (−25%) in ED rats than in C rats. The total cost of storage was determined taking into account that the energy loss in storing 1 kJ of protein is 1.25 kJ (Pullar & Webster, 1977), while the corresponding energy cost for fat deposition is 0.36 kJ/kJ for diets with a high percentage of carbohydrates, such as the control diet, and 0.16 kJ/kJ for diets with a high fat content, such as the energy-dense diet (Pullar & Webster, 1977). The values obtained for energy expenditure excluding the total cost of storage, called net energy expenditure (NEE), are reported in the same table and were significantly higher (+131%) in ED rats than in C rats. When NEE was expressed as a percentage of ME, a significant increase (+77%) was found in ED rats compared with C rats (Table 2).

The O<sub>2</sub> consumption in isolated hepatocytes from C and ED rats was measured under different conditions of stimulation of mitochondrial respiration (Fig. 1). When the cells were provided with additional substrates, namely hexanoate, glycerol or sorbitol, a significant increase in O<sub>2</sub> consumption for the two groups occurred in comparison with respective basal states (hepatocytes incubated without added substrates). Liver cells from

Table 2. Energy balance in rats fed on a control (C) diet or an energy-dense (ED) diet  
(Mean values with their standard errors for five rats over a 15 d period)

Diet...	C		ED	
	Mean	SE	Mean	SE
Initial body wt (g)	75	2	75	2
Final body wt (g)	190	4	189	2
Body-wt gain (g)	115	2	114	2
Carcass lipid content (g/kg)	114	5	119	8
Carcass protein content (g/kg)	224	5	205	14
Metabolizable energy (ME) intake (kJ)	2547	165	3320*	111
Body energy gain (kJ)	1012	56	1000	55
Energy expenditure (kJ)	1535	163	2320*	165
Gross efficiency† %	40	3	30*	3
Storage cost, protein‡ (kJ)	572	62	461	23
Storage cost, fat§ (kJ)	199	15	93*	8
Total cost of storage (kJ)	771	69	554*	22
Net energy expenditure   (NEE) (kJ)	764	52	1766*	175
NEE/ME intake × 100	30	4	53*	3

\*Mean values were significantly different from those for controls,  $P < 0.05$  (two-tailed Student's  $t$  test).

†(Body energy gain/ME intake) × 100.

‡Value of 1.25 kJ/kJ was used to estimate the storage cost of protein.

§Values of 0.36 kJ/kJ (control diet) and 0.16 kJ/kJ (energy-dense diet) were used to estimate the storage cost of fat.

||Energy expenditure excluding the total cost of storage.

ED rats, incubated with or without added substrates, exhibited a significantly higher  $O_2$  consumption than those from C rats.

Fig. 1 also shows the effect of ouabain on hexanoate-supported respiration. The decrease in  $O_2$  consumption due to the specific Na/K-ATPase (*EC* 3.6.1.37) inhibitor, ouabain, was significantly higher in ED rats than in C rats. Therefore, in ED rats the Na/K-pump-dependent  $O_2$  consumption increased significantly (+ 148 %).

Serum triacylglycerol concentrations were 1.33 (SE 0.10) and 2.00 (SE 0.10) mM in C and ED rats respectively, and FFA concentrations were 0.60 (SE 0.03) and 0.83 (SE 0.02) mM in C and ED rats respectively. Concentrations of both variables were significantly higher (+ 50 % and + 38 % respectively) in ED rats than in C rats.

## DISCUSSION

Rats fed on the energy-dense diet showed increases in ME intake, energy expenditure and NEE (Table 2). When the NEE was expressed as a percentage of ME the values obtained in the two groups were significantly different. This finding indicates that the NEE : ME ratio is not constant whatever the energy intake and suggests the presence of regulatory mechanisms controlled by ME intake, which are useful to limit fat gain. The last result is different from those obtained by LeBlanc *et al.* (1986), who found no variation in NEE : ME ratio in rats fed on a cafeteria diet.

In the present study we have also found that feeding rats on the energy-dense diet resulted in significant increases in liver respiratory activity (Fig. 1). The respiration values of isolated hepatocytes indicate that  $O_2$  consumption measured without exogenous substrates depends not only on the ATP : ADP ratio, as is generally believed (Schwenke *et al.* 1981; Soboll & Stucki, 1985), but also on the supply of substrate to the electron

transport chain (Nobes *et al.* 1990). In fact, the addition of substrates which generate reducing equivalents into the mitochondria (hexanoate) or into the cytoplasm (glycerol and sorbitol) increased endogenous respiration in both groups of rats (Fig. 1). On the other hand, liver respiration with added substrates is essentially controlled by the intracellular ATP:ADP ratio; consistent with this, we have shown previously that the addition of lactate, which stimulates gluconeogenesis and hence ATP hydrolysis, causes a further increase in respiration (Iossa *et al.* 1991). The results obtained for liver respiration in ED rats show a significant increase both in basal state (without exogenous substrate) and in the presence of added substrates (Fig. 1). These results are in agreement with previous ones obtained with hepatocytes from rats fed on a cafeteria diet (Berry *et al.* 1985). The observed increase in basal respiration may be due to an improvement in substrate supply, which would be consistent with the increase in serum FFA concentrations found in ED rats. On the other hand, the increase in liver respiration in the presence of added substrates may be due to a greater ADP availability. In line with this, our results show that the Na/K-pump-dependent O<sub>2</sub> consumption was significantly higher in ED rats than in C rats (Fig. 1). It should be noted that about 57 % of the increased hepatocyte respiration in ED rats is directly attributable to increased Na pumping activity, which in turn elicits a greater liberation of metabolic heat. This increased heat production is in good agreement with the increased energy expenditure found in ED rats (Table 2).

Additional metabolic implications could come from the observation of the increase in hepatic respiration not only with glycerol and sorbitol, whose oxidation is known to be linked mainly to FADH<sub>2</sub> oxidation (Berry *et al.* 1973), but also with hexanoate, whose oxidation is strictly dependent on the mitochondrial NADH levels (Sherratt & Spurway, 1994). In fact, taking into account the impairment of NADH-dehydrogenase (EC 1.6.5.3) previously found in mitochondria isolated from ED rats (Iossa *et al.* 1995), the present increase in hepatic respiration is in line with our previous hypothesis (Iossa *et al.* 1995). Based on the increase in mitochondrial NADH:NAD ratio which occurs in ED rats (Iossa *et al.* 1995), we have suggested a shift of the reaction catalysed by malate dehydrogenase (EC 1.1.1.37) in favour of malate production at the expense of oxaloacetate; this in turn favours the export of malate to the cytosol and its conversion to pyruvate or oxaloacetate, with the formation of NADPH or NADH. The reducing equivalents can be finally retransported into the mitochondria through the  $\alpha$ -glycerophosphate shuttle, the activity of which increases in ED rats (Iossa *et al.* 1995), and oxidized through the respiratory chain from complex II onwards. The mechanism described would thus lead to a fall in hepatic metabolic efficiency, with a subsequent wasteful increase in the oxidation of energetic substrates, such as fatty acids. Another possibility is that reducing equivalents are oxidized in the cytosol because of an increased utilization of gluconeogenic substrates which do not generate NADH (alanine), at the expense of those that generate NADH (lactate).

In conclusion, the increased hepatic respiratory activity found in ED rats may represent one of those regulatory mechanisms which are useful to counteract development of obesity.

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